

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

At pages 5, please amend the specification as follows:

**Brief Description Of Figures.**

FIGURE 1 shows images of ~~in~~ primary neuronal cultures from the subthalamic nucleus infected with AAV virus vectors expressing GAD-67 (top two panels), or virus vectors expressing GAD-65 (middle two panels). The bottom two panels show cells infected with the GAD-65 plasmid (left bottom panel) and the GAD-67 plasmid (right bottom panel).

FIGURES 1A - 1F 2A - 2F are microphotographs showing plasmid transfection according to the invention; FIGURES A and D show plasmid transfection of HEK 293 cells with 1 $\mu$ g of rAAV DNA and FIGURES B and E. show rAAV vector transduction of HEK 293 cells with S $\mu$ l rAAV vector while FIGURES C and F. show non-transfected HEK 293 cells.

FIGURE 2 3 is a graph showing the effect of rAAV transduction on the GABA release of primary cultured striatal neurons;

FIGURE 3 4 is a graph showing the effect of rAAV-GAD treatment on apomorphine-induced rotation in chronic Parkinson's Disease Rats;

FIGURE 4 5 is a graph showing the neuroprotective effect of rAAV-GAD treatment on apomorphine-induced rotation;

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

**At pages 6, please amend the specification as follows:**

FIGURE 5A 6A is a graph showing the potent neuroprotective effect of GAD65 on apomorphine rotation:-;

FIGURE 5B 6B is another graph showing the potent neuroprotective effect of GAD65 on apomorphine rotation:-;

FIGURE 6A 7A is a graph showing that there was no significant reduction in head position bias 2 months after rAAV transduction in chronic Parkinson's Disease Rats:-;

FIGURE 6B 7B is a further graph showing that there was no significant reduction in head position bias 4 months after rAAV transduction in chronic Parkinson's Disease Rats:-;

FIGURE 7A 8A is a graph demonstrating that head position bias was improved in rats transduced with rAAV-GAD65:-;

FIGURE 7B 8B is a further graph showing that rAAV-GAD65 transduced rats showed marked effects on head position bias:-;

FIGURE 8 9 is a graph demonstrating a direct correlation between apomorphine rotation and head position bias:-;

FIGURE 9 10 is graph showing that paw touching counts were significantly improved in all rAAV-GAD and Ibotenic acid lesion groups:-;

FIGURE 10 11 is a further graph showing that rAAV-GAD-65 had a marked neuroprotective effect on paw touching counts:-;

FIGURE 11A 12A is a graph demonstrating that a marked improvement in locomotor activity was observed in Parkinson's Rats with combined rAAV-GAD65 and 67:-;

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

**FIGURE 11B 12B** is another graph further demonstrating that a marked improvement in locomotor activity was observed in Parkinson's Rats with combined rAAV-GAD65 and 67A

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

At pages 7, please amend the specification as follows:

FIGURE 12A 13A is a graph showing that there was also evidence of neuroprotective effects on locomoter activity by rAAV-GAD transduction;

FIGURE 12B 13B is a graph further showing a neuroprotective effects on locomoter activity by rAAV-GAD transduction;

FIGURE 13 14 is a graph of extracellular GABA Concentration during STN Stimulation;

FIGURE 14 15 is a graph of Extracellular Glutamate Concentration during STN Stimulation;

FIGURE 15 16 is a histogram showing the response of neurons in the Substantia Nigra to electrical stimulation in the STN of a normal rat;

FIGURE 16 17 is a histogram showing the response of neurons in the Substantia Nigra to electrical stimulation in the STN in rAAV-GAD transduced rat;

FIGURE 17A 18A is a graph of extracellular GABA concentration in the SN during STN stimulation in naïve rats;

FIGURE 17B 18B is a graph of extracellular GABA concentration in the SN during STN stimulation in rAAV-GAD rats;

FIGURE 18A 18F 19A – 19F are microphotographs showing rAAV-GAD65 expression in vivo. FIGURES 18 19 A,B,C, and D show GAD65 expression in the STN detected with GAD65 Ab (Boehringer). FIGURES 18A 19A and C are derived from naïve STN, showing endogenous GAD65 expression. FIGURES 18B 19B and D are based on rAAV-GAD65 transduced STN, such that an increase in cell bodies expressing GAD65 is seen, while FIGURES 18 19 E and F show GAD65 expression in the hippocampus. (FIGURE 18E 19E being naïve and FIGURE 18F 19F being rAAV-GAD65 transduced);

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

FIGURES 19A and 19B 20A and 20B are rasterplots showing activity in a monkey before GAD67 treatment, respectively;

~~FIGURE 20 is a microphotograph FIGURES 21A and 21B are microphotographs showing GFP immunostaining at a an injection site. Neuronal-like cells stained with GFP antibody are shown in 21A and glial-like cells stained with GFP antibody are shown in 21B;~~

22

~~FIGURES 21A and 21B are FIGURE 21 is a more detailed images, image of GFP immunostaining at an injection site showing neuronal-like cells stained with GFP antibody in 21A 22A and glial-like cells stained with GFP antibody shown in 21B.; and~~

~~FIGURE 22 23 is a photograph of GAD immunostaining on rAAV-GAD treated monkey, showing an increase in immunostaining on the rAAV-GAD treated side on the right while the morphology of the region remained unaltered after surgery.~~

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

At page 27, please amend the second full paragraph as follows:

***Example 2: In vitro transduction of the AAVGAD vectors***

The GAD-65 and GAD-67 vectors were transduced into primary neuronal cultures from the subthalamic nucleus. Fig. 4 FIG. 1 shows an image of cells infected with AAV vectors expressing GAD-67 (top two panels) with a MOI of 10 (multiplicity of infection) in transient transfection experiments. The antibodies were detected using a commercially available antibody for Immunocytochemical detection. A similar experiment was conducted using cells infected with AAV vectors expressing GAD-65 with an MOI of 10 (middle two panels), and detected using an antibody specific for GAD-65. This data demonstrates successful transduction of vectors and successful expression of the vectors *in-vitro* in primary neuronal cultures from the subthalamic nucleus.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

**At page 35, please amend the second full paragraph as follows:**

***Results***

The results showed that GAD65/GAD67 expression was detected after plasmid transfection and virus transduction of HEK 293 cells. No GAD65 or GAD67 was detected in untransfected or untransduced cells. ~~Fig. 1A and Fig. 1D~~ FIG. 2A and FIG. 2D show plasmid transfection of HEK 293 cells with 1 $\mu$ g of rAAV DNA. ~~Fig. 1B, Fig. 1B~~ FIG. 2B, FIG. 2E show rAAV vector transduction of HEK 293 cells with 5 $\mu$ l rAAV vector. ~~Fig. 1C and Fig. 1F~~ FIG. 2C and FIG. 2F shows non-transfected HEK 293 cells.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

At page 36, please amend the first full paragraph as follows:

*Example 5: GABA Release from Primary Cultured Striatal Neurons Transduced with  
rAAV-GAD Vectors*

Primary striatal cultures were prepared from day 15 embryos and plated onto poly-L-lysine coated wells of a 24 well plate at a density of  $2.5 \times 10^5$  for striatal culture and 48 hours later, 21 of the following viruses was added to each well in triplicate:

AAV/CB-hGAD65-WPRE

AAV/CB-hGAD67-WPRE

AAV/CB-EGFP-WPRE (control virus).

Ten days later the cells were washed five times in PBS then incubated 5 min in 200 $\mu$ l aCSF. (first wash). This was collected then the cells were incubated in 200  $\mu$ l aCSF+ 56mM KCl for 10 mins at 37°C (high K+). HPLC was performed to determine the amount of GABA released.

The results demonstrated that both GAD67 and GAD65 expression significantly increased the basal and K+-induced release of GABA compared to GFP control (see Fig. 2 FIG. 3).

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

At page 44, please amend the first and second full paragraphs as follows:

Apomorphine-induced rotational asymmetries

In the chronic Parkinson's Disease study, rAAV-GAD treatment groups showed reduced rotations under apomorphine compared to the progressive PD group, which was similar to the ibotenic acid lesioning of STN. Fig. 3 FIG. 4 is a graph showing the effect of rAAV-GAD treatment on apomorphine-induced rotation in chronic Parkinson's Disease Rats.

In neuroprotective study, all rats administered rAAV-GAD65/67 showed protection against 6-OHDA insult. Fig. 4 FIG. 5 is a graph showing the neuroprotective effect of rAAV-GAD treatment on apomorphine-induced rotation. Rats with rAAV-GAD65 showed the best protective effect, over 69% rats showed absolutely no rotational asymmetry. Figs. 5a and 5b FIGS. 6A and 6B are graphs showing the neuroprotective effect of rAAV-GAD treatment on apomorphine-induced rotation. Collectively, this data shows that GAD65 and GAD67 injected animals displayed a decrease in apomorphine induced rotations over 15-20 mins.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

**At page 45, please amend all four paragraphs as follows:**

The 6-OHDA lesion induced ipsilateral bias. This was used as one the quantitative markers of the parkinsonian phenotype. No significant reduction in 6-OHDA lesion induced ipsilateral head position bias was observed in a rAAV-GAD65, 67 or 65 and 67 administered chronic hemiparkinsonian rats (Figs. 6a and 6b) (FIGS. 7A and 7B). However, in rats with rAAV-GAD65, this symmetry bias was much improved (Figs. 7a and 7b) (FIGS. 8A and 8B). The GAD67 group was not tested at 14 weeks. Fig. 8 FIG. 9 is a chart showing there is a direct correlation between apomorphine rotation and head position bias.

#### **Paw touching**

The 6-OHDA lesion induced a decreased forepaw rising and touching movement as well as an ipsilateral bias. Forepaw touching movement was significantly improved in all rAAV-GAD and ibotenic acid lesion groups of Chronic PD rats. Fig. 9 FIG. 10 is a chart showing paw touching counts were significantly improved in all rAAV-GAD and ibotenic acid lesion groups. The GAD65/67 group was not tested at 14 weeks. Prior administration of rAAV-GAD65 effectively protected against the loss of paw touching movement induced by MFB 6-OHDA lesioning. Fig. 10 FIG. 11 is a chart showing rAAV-GAD-65 had a marked neuroprotective effect on paw touching counts.

#### **Locomotor Activity**

The horizontal locomotor activity decreased progressively in chronic Parkinson's rats. Combined rAAV-GAD65 and 67 transduced rats showed marked improvements in their locomotor function. Figs 11a and 11b FIGS. 12A and 12B are graphs showing a marked improvement in locomotor activity was observed in Parkinson's Rats with combined rAAV-GAD65 and 67.

Prior administration of rAAVGAD65 also protected effectively against the reducing horizontal locomotor activity induced by MFB 6-OHDA lesion. Figs. 12a and 12b FIGS. 13A

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

and 13B are charts showing there was evidence of neuroprotective effects on locomoter activity by rAAV-GAD transduction.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

**At page 46, please amend second full paragraph as follows:**

Inhibitory GABA containing connections were detected from the STN to the SN using electrophysiology and microdialysis. In the microdialysis experiments, a 10X increase in GABA was detected due to low frequency electrical stimulation of the STN, compared to a 3X increase in control rats. Table 4 for GAD rat #304 and for control rat # 217 shows the concentration of GABA, glutamate and aspartate in the SN obtained before and after low frequency stimulation. The sample labels are Basal #, for the samples taken before stimulation, ST1 - #, for successive samples after the first low frequency stimulation for 2 minutes and ST2 - #, for successive samples after the first low frequency stimulation for 5 minutes. ~~Figs 13 and 14~~ FIGS 14 and 15 are charts showing extracellular GABA concentration during STN stimulation and correspond to the GABA and glutamate data in Table 4.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

**At page 47, please amend first full paragraph as follows:**

Figs. 15 and 16 FIGS. 16 and 17 show the response of neurons in the Substantia Nigra (SN) to electrical stimulation of the STN. These Figures show a histogram (20 ms bins) of spike counts after a electrical stimulation at  $t = 0$ . Each trial of the stimulation used to create the histogram is included and labeled sweep of the graph. Fig. 15 FIG. 16 is a chart showing the response of neurons in the Substantia Nigra to electrical stimulation in the STN of a normal rat and shows that in normal rats there is a large increase in impulse activity due to STN stimulation. Fig. 16 FIG. 17 is a chart showing the response of neurons in the Substantia Nigra to electrical stimulation in the STN in rAAV-GAD transduced rat and shows an inhibition of spontaneous firing of the neuron in the SN due to STN stimulation. The stimulation in each of Figs. 15 and 16 FIGS. 16 and 17 occurred at time = 0. The histograms and raster plots shows 200ms before and 800ms after the stimulus for comparison of the impulse rate immediately after stimulation.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

At page 48, please amend first and second full paragraphs as follows:

*iii) Extracellular GABA and Glu concentrations in Substantia Nigra Microdialysis during STN Stimulation*

The current data show a significant increase in extracellular GABA in GAD65 transduced compared to naïve rats following low frequency stimulation of the STN. There was a 4.4x increase in mean GABA concentration during the first 15 min fractions after the LFS in GAD65 transduced group, compare to a 1.5x increase in naïve control. An increasing extracellular glutamate was also observed in both naïve and GAD65 transduced rats. Fig. 17a FIG. 18A is a chart showing extracellular GABA concentration in the SN during STN stimulation in naïve rats (N=4). Fig. 17b FIG. 18B is a chart showing extracellular GABA concentration in the SN during STN stimulation in rAAV-GAD rats (N=3) NB. ST1 – 2 min Low Freq Stim ST2-5 min Low Freq Stim.

Fig. 18A-F FIG. 19A-F is a photograph showing AAV-GAD65 expression in vivo in naïve and GAD65 transduced animals. A,B,C, and D; GAD65 expression in the STN detected with GAD65 Ab (Boehringer). A and C; Naïve STN, showing endogenous GAD65 expression. B and D; rAAV-GAD65 transduced STN, an increase in cell bodies expressing GAD65 is seen. E and F; GAD65 expression in the hippocampus. E; naïve. F; rAAV-GAD65 transduced.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

At page 55, please amend second full paragraph as follows:

*iii) Activity*

Prior to any treatment, spontaneous general activity levels in the home cage measured with personal activity monitors located in primate jackets were similar to what was observed in previous studies. As observed in the clinical rating, after MPTP treatment the animals presented variable activity levels during the day. Figs. 19A and 19B FIGS. 20A and 20B are rasterplots showing activity before (A) and after (B) GAD67 treatment (monkey 6482). Observe the presence of hills and valleys corresponding to the activity during the day and night respectively. In all the cases, a circadian rhythm was observed and remained unaffected after AAV surgery (Fig. 19 FIG. 20).

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

**At page 57, please amend first full paragraph as follows:**

rAAV-GFP treated monkeys (6436 and 6442) presented GFP positive cells limited to the subthalamic nucleus ipsilateral to the rAAV injection. The cell bodies were easily identified and limited in number to 6-10 positive neuron-like cells per animal. In contrast, no monkeys receiving rAAV-GAD presented GFP positive cells. ~~Figs. 20A and 20B~~ FIGS. 21A and 21B are photographs of GFP immunostaining at an injection site (GFP antibody from Clontech Palo Alto California). Fig. 21 is a more detailed image of Fig. 16, showing neuronal-like cells stained with GFP antibody in (A), while glial-like cells stained with GFP antibody are shown in (B). FIG. 22 is a more detailed image of GFP immunostaining at injection site.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

**At page 57, please amend third full paragraph as follows:**

In comparison, rAAV-GAD treated animals showed increase GAD staining in the subthalamic nucleus ipsilateral to the AAV injection. Rh 6474 (GAD65) presented only a mild increase of GAD positive fibers. However, Rh 6485 (GAD67) displayed robust expression of GAD distributed throughout the neuropil of the subthalamic and immediately adjacent area. Fig. 22-FIG. 23 is a photograph of GAD immunostaining on rAAV-GAD treated monkey. There is an increase in immunostaining on the rAAV-GAD treated side on the right. The morphology of the region remained unaltered after surgery.

Application No: 09/863,179  
Filing Date: May 23, 2001  
Group Art Unit: 1632  
Examiner: Falk, A. M.  
Attorney Docket No: 102182-12

The Examiner is urged to call the undersigned at the telephone number indicated below so that any remaining issues can be discussed.

Respectfully submitted,  
NUTTER, McCLENNEN & FISH, LLP

Date: March 11, 2004

  
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